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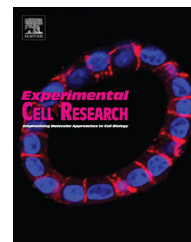
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Research Article

Basement membrane influences intestinal epithelial cell growth and presents a barrier to the movement of macromolecules



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ABSTRACT

This work examines the potential drug delivery barrier of the basement membrane (BM) by assessing the permeability of select macromolecules and nanoparticles. The study further extends to probing the effect of BM on intestinal epithelial cell attachment and monolayer characteristics, including cell morphology. Serum-free cultured Caco-2 cells were grown on BM-containing porous supports, which were obtained by prior culture of airway epithelial cells (Calu-3), shown to assemble and deposit a BM on the growth substrate, followed by decellularisation. Data overall show that the attachment capacity of Caco-2 cells, which is completely lost in serum-free culture, is fully restored when the cells are grown on BM-coated substrates, with cells forming intact monolayers with high electrical resistance and low permeability to macromolecules. Caco-2 cells cultured on BM-coated substrates displayed strikingly different morphological characteristics, suggestive of a higher level of differentiation and closer resemblance to the native intestinal epithelium. BM was found to notably hinder the diffusion of macromolecules and nanoparticles in a size dependent manner. This suggests that the specialised network of extracellular matrix proteins may have a significant impact on transmucosal delivery of certain therapeutics or drug delivery systems.

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Introduction

Cell culture-based in vitro intestinal epithelial models are used in a wide variety of research disciplines ranging from study of oral drug delivery [1–3] and nutrient transport [4,5] to investigations into entry mechanisms of infectious agents [6,7] and intestinal disease processes [8,9]. Whilst these models offer advantages,

including controlled conditions and reduced animal experiments, current epithelial models fail to accurately represent the native intestinal epithelium. Intestinal epithelial models are currently based on culture of a suitable cell type, predominantly Caco-2, directly on flat, porous supports. However, such culture conditions fail to accurately reflect the environment of the native intestinal epithelium, where epithelial cells are supported by the

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basement membrane (BM) as a specialised form of extracellular matrix (ECM).

The intestinal BM is a thin network of ECM, composed of a number of proteins, particularly laminins, type IV collagen and fibronectin [10–12]. These proteins play an important role in biological processes of intestinal cell migration, proliferation, and differentiation [13,14]. The response and behaviour of intestinal epithelial cells following direct culture on flat, polymer-based supports may therefore not be reproduced due to the lack of the natural interaction between the cells with the natural microenvironment in the form of the BM. Consequently, the resulting in vitro model may fail to achieve appropriate characteristics, including cell morphology, expression of relevant proteins and barrier properties, which clearly presents reliability issues for the model. Employing more biologically relevant conditions for culture of intestinal epithelial cells is therefore desirable as the resulting model may express a more relevant phenotype and characteristics.

At the same time, the extent to which the BM contributes to the overall mucosal barrier in terms of mucosal absorption of material is unclear, in contrast to the drug delivery barrier of the components of the mucosa other than the BM, including mucus and the epithelial cell layer, which have been relatively well researched [15,16]. This is particularly important considering the increasing efforts to enable effective non-invasive, mucosal delivery of macromolecular biotherapeutics as BMs have been shown to hinder the diffusion of macromolecules as small as 5000 Da in the non-keratinized oral mucosal epithelium [17]. With an increasing proliferation of biotherapeutics and nanomedicines, coupled with the desire to achieve oral delivery in particular, there is a need to study the drug delivery barrier of the intestinal mucosal BM.

Here we set out to investigate the barrier property of the BM with respect to the movement of macromolecules and nanoparticles as increasingly relevant carriers of therapeutics [18–20]. In doing so, we employed a simple method to obtain BM coated supports on which we cultured Caco-2 cells. This modification resulted in a significant difference on cell attachment, morphology and barrier characteristics of these epithelial monolayers. In this paper we report both the characterisation of these effects on the intestinal epithelial Caco-2 cells and also on the barrier characteristics of both the BM and the epithelial monolayer.

Materials and methods

Materials

Calu-3 bronchial adenocarcinoma cells (used between passages 19–48) and Eagle's Minimal Essential Medium (EMEM) were obtained from the American Type Culture Collection (ATCC)-LGC Promochem (USA). Caco-2 colorectal adenocarcinoma cells (passages 44–58) were obtained from European Collection of Cell Cultures (ECACC). Ham's F-12 medium, Dulbecco's Modified Eagle's Medium (DMEM), Hank's Balanced Salt Solution (HBSS, with sodium bicarbonate and without phenol red), non-essential amino acids, L-glutamine, antibiotic/antimycotic solution (10–12,000 U/ml penicillin, 10–12 mg/ml streptomycin, 25–30 µg/ml amphotericin B), 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid solution (HEPES), non-animal recombinant trypsin solution

(TrypZan[®]), soybean trypsin inhibitor, and fluorescein isothiocyanate (FITC)-labelled dextran of approximate molecular weight of 4 and 10 kDa (FD4 and FD10, respectively) were all supplied by Sigma-Aldrich (UK). Transwell[®] permeable inserts of 12 mm diameter and 0.4 µm pore size (referred to as 'porous supports' or simply, 'substrates') were purchased from Corning (USA). Mouse, anti-human laminin $\alpha 3$ /laminin-5 ($\alpha 3\beta 3\gamma 2$) antibody (monoclonal mouse IgG1), derived from an established in vitro hybridoma, was obtained from R&D Systems (USA). Mouse, anti-human zonula occludens-1 (ZO-1, tight junction protein) and Epilife[®] Calu-3 serum free (and chemically defined) medium were purchased from Life Technologies Corp. (UK). Goat, anti-mouse IgG-Atto 488 (secondary antibody) was obtained from Sigma-Aldrich (UK). FITC-labelled human serum albumin was supplied by Abcam (UK). Recombinant human insulin solution, human transferrin, sodium selenite, human serum albumin (HSA) were all purchased from Sigma-Aldrich (UK). Fluorescent, carboxylate-modified polystyrene nanoparticles of 20 nm (FluoSpheres[®]; 505 nm excitation, 515 nm emission) and 50 nm diameters (Fluoresbrite[®]; 441 nm excitation, 486 nm emission) were purchased from Life Technologies Corp. (UK) and PolySciences Inc. (Germany). Collagenase from *Clostridium histolyticum*, Quanti-Pro[™] high sensitivity BCA (bicinchoninic acid assay) kit, IgG-FITC from human serum, human placenta type IV collagen, human plasma fibronectin and all other chemicals were supplied by Sigma-Aldrich (UK).

Cell culture

Calu-3 and Caco-2 cells were gradually transferred from serum-containing media to serum-free, chemically defined media by reducing the proportion of serum-containing medium and increasing that of the serum-free medium at each subculture point. Caco-2 cells were eventually transferred from 10% v/v DMEM to serum-free and chemically defined Ham's F-12, whilst Calu-3 cells were gradually converted from EMEM to serum free, chemically-defined Epilife[®] (with added supplement S7). Ham's F-12 was supplemented with 6.25 µg/ml human recombinant insulin, 6.25 µg/ml human transferrin, 1.25 mg/ml human recombinant albumin, 6.25 ng/ml selenium and 5.35 µg/ml linoleic acid, which is a slightly modified culture medium 'recipe' to that previously reported to allow serum-free culture of Caco-2 cells [21].

Unless otherwise stated, culture on permeable supports was conducted using serum-free media for both airway Calu-3 and intestinal Caco-2 cell lines. Both cell lines were seeded on permeable supports at 10^5 cells/cm². For Calu-3 cells, air-interface culture (AIC) conditions were created on day 2 post-seeding (culture medium removed from the apical side) and cells were thereafter cultured using AIC conditions. Culture medium was replaced every 2–3 days for both cultures.

Expression of laminin-5 by Calu-3 cells

Calu-3 cells were cultured on permeable supports as described above. Prior to the immunostaining procedure, the resulting cultures were tested for formation of confluent and polarised layers by measurement of transepithelial electrical resistance (TEER). Only cells displaying a TEER of $> 500 \Omega \text{cm}^2$ were included in the immunostaining studies. Culture medium was removed and

cells washed with PBS. Cells were then fixed with 4% (w/v) paraformaldehyde (in PBS; 10 min incubation) and permeabilised using Triton X-100 (0.1% v/v in PBS; 10 min incubation). Cells were then incubated with human serum albumin in PBS (1% w/v) for 1 h, followed by incubation with mouse, anti-human laminin α 3/laminin-5 antibody (10 μ g/ml in 1% HSA/PBS) for another hour. Following extensive washing, cells were incubated with goat, anti-mouse IgG-Atto 488 at 5 μ g/ml for 1 h. Cell samples were then washed and Transwell® membranes excised and mounted on glass slides using a DAPI-containing mounting medium. Cells were imaged using a Leica TCS SP2 system mounted on a Leica DMIRE2 inverted microscope (Germany).

Substrate deposition of basement membrane proteins by Calu-3 cells

Protein deposition. Calu-3 cells were cultured on Transwell® permeable supports (in AIC conditions) for 5 or 19 days. A decellularisation step was then conducted by washing the cells with PBS, followed by 10–15 min incubation with 0.02 M ammonium hydroxide. Transwell® supports were then washed extensively with PBS to ensure complete cell removal. To desorb protein for quantitation, the permeable supports were incubated with 8 M NaCl, for 1–2 h followed by protein quantitation using a high sensitivity BCA kit. Calibration curves, employing a series of known concentrations of human serum albumin were used to convert the absorbance readings of the samples into concentrations.

Immunofluorescence. Calu-3 cells were cultured on permeable supports for 5 days and decellularisation carried out as before. The supports were then washed extensively with PBS, followed by a 1-h incubation with 1% w/v HSA/PBS. Thereafter, mouse, anti-human laminin α 3/laminin-5 IgG was applied at 10 μ g/ml for 1 h. The primary antibody was then removed and the plastic support washed extensively with PBS. Goat, anti-mouse IgG-Atto 488 was then applied at 5 μ g/ml and the supports incubated at room temperature for 1 h. The substrates were washed again with PBS, fixed with paraformaldehyde, excised and finally mounted on glass slides for confocal imaging. Cells were imaged using a Leica TCS SP2 system mounted on a Leica DMIRE2 inverted microscope.

Caco-2 culture on decellularised substrates

Calu-3 cells were seeded and cultured on Transwell® permeable supports using the AIC conditions, described above ('Cell culture' section), for 5–7 days. Supports were then decellularised using 0.02 M ammonium hydroxide, as described above (section 'Substrate deposition of basement membrane proteins by Calu-3 cells'). Permeable supports were then washed extensively with PBS, and Caco-2 growth medium (Ham's F-12, with supplements) applied on both donor and acceptor compartments of the Transwell® system. Caco-2 cells were then seeded at 10^5 cells/cm² and cultured for 21 days. Caco-2 cell medium was changed at regular intervals (2–3 times weekly). A control experiment was conducted in parallel where Caco-2 cells were seeded on permeable supports previously exposed to Calu-3 culture medium (Epilife®) and treated with 0.02 M ammonium hydroxide to replicate the conditions used for Caco-2 culture on decellularised substrates. This was done to test any effects that the culture medium or the decellularisation step may have on Caco-2 attachment/growth.

Caco-2 culture on type IV collagen and fibronectin-coated supports

Permeable supports were coated with ECM/BM proteins known to enhance cell attachment, namely human type IV collagen and human fibronectin. Coating with type IV collagen was conducted following the supplier's instructions, using 10 μ g collagen (dissolved in 0.25% v/v acetic acid) per support (equivalent to 9.1 μ g/cm²). Transwell® permeable supports were incubated overnight at 2–8 °C to allow surface adsorption. Dried, coated dishes were then sterilized by rinsing with 70% v/v ethanol. Supports were then washed with PBS before cell seeding. Human fibronectin coating was achieved following the manufacturer's protocol, by treating the Transwell® supports with a solution providing 2.5 μ g/cm² fibronectin and incubating at room temperature for 1 h. The solution was then aspirated and supports rinsed with cell culture grade water, whilst avoiding scraping the support surface. Seeding and culture of Caco-2 cells on these supports was conducted in the same way as seeding/culture on decellularised supports (i.e. 10^5 cells/cm² and Ham's F-12 medium with supplements).

Chemical and enzymatic treatment of decellularised substrates

Calu-3 culture on permeable supports and decellularisation was performed as described previously (see section 'Caco-2 culture on decellularised substrates'). Following the decellularisation step, permeable supports were subjected to a 1-h treatment with the following chemicals and enzymes: 4 M guanidine hydrochloride, 0.05 M NaOH, 1 mg/ml collagenase and trypsin (non-animal recombinant trypsin, TrypZean®).

Characterisation of Caco-2 monolayer cultured on decellularised substrates

TEER

Caco-2 cells were cultured on permeable Transwell supports previously used for Calu-3 culture, followed by a decellularisation step, as described in the preceding section. TEER measurements were conducted periodically, at times when culture medium was replaced (measurements were taken prior to medium replacement).

In a control experiment, Caco-2 cells were seeded on permeable supports that were previously exposed to Epilife medium only (i.e. Calu-3 cells were not cultured on the supports). Furthermore, to test the effect of cell attachment-mediating proteins collagen and fibronectin (ECM proteins) or serum, Caco-2 cells were cultured on transwells treated with these components under the same conditions (10^5 cells/cm² seeding density and Ham's F-12 chemically-defined medium).

TEER is expressed as Ω cm² in all cases, with the reported values accounting for background TEER resulting from the Transwell plastic.

Permeability

Caco-2 cells were cultured on decellularised permeable supports for a period of 21 days. Prior to the permeability experiment, cell monolayer TEER was measured to ensure monolayer intactness. Cell medium (Ham's F-12) was then replaced with HBSS;

cells were subsequently incubated with HBSS for approximately 45 min. FITC-dextran of approximately 4 kDa and 10 kDa (FD4 and FD10, respectively), dissolved in HBSS at 500 µl/ml were then applied to the apical side of the cell monolayers. FD4 permeability was determined by sampling the basolateral solution periodically (every 30 min) for 3 h, whilst replacing the sampled volume with fresh HBSS at each interval. FD4 was quantified by fluorescence (using calibration curves) and apparent permeability coefficient (P_{app}) calculated using the following equation:

$$P_{app} = \left(\frac{\Delta Q}{\Delta t} \right) \times \left(\frac{1}{A \times C_0} \right)$$

where P_{app} is the apparent permeability in cm/s, $\Delta Q/\Delta t$ is the permeability rate (amount FD4 or FITC-insulin traversing the cell layers over time, as determined from the steady state accumulation of the solute in the receiver chamber over time), A is the surface area of the cell layer (1.1 cm²) and C_0 is the initial FD or FITC-insulin concentration in the donor chamber.

Scanning electron microscopy (SEM)

Caco-2 cells were cultured on permeable supports for 21 days, following Calu-3 decellularisation, as described in section 'Caco-2 culture on decellularised substrates'. Cells were fixed initially with a 1:1 mixture of culture medium and fixing solution, which comprised 2.5% v/v glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) on both apical and basolateral sides of the Transwell. Following an incubation interval of 5 min, this solution was removed and replaced with 100% of the fixing solution. The fixing solution was then removed and replaced with 1% w/v osmium tetroxide in water; cell samples were incubated with this solution at room temperature for 90 min. Osmium tetroxide solution was then removed and samples dehydrated in progressively increasing concentrations of ethanol in water (25%, 50%, 75%, 95% and 100% v/v). Samples were dried using a critical point dryer before filters were removed and mounted on aluminium stubs with adhesive double-sided carbon tape. The samples were gold coated for 3 min under an argon atmosphere in a Blazers Union SCD030 sputter coater unit (Blazers Ltd, UK). Coated samples were examined with a JEOL 6060LV (JEOL, Welwyn, UK) variable pressure scanning electron microscope operating at an accelerating voltage of 10 kV. Image analysis was carried out using the in-built SEM control user interface software (version 6.57) and digital imaging system.

Tight junction immunostaining

Caco-2 cells were cultured on porous supports for 21 days after Calu-3 growth and decellularisation. Tight junction staining was performed by initially fixing the cells with paraformaldehyde, followed by washing with PBS and permeabilisation with Triton X-100 (0.1% v/v in PBS). Cells were then washed with PBS, followed by the application of 1% w/v human serum albumin (HSA)/PBS for 1 h. Thereafter, HSA /PBS solution was replaced with mouse, anti-human ZO-1 (primary) antibody, diluted in 1% w/v HSA/PBS to a final concentration of 10 µg/ml. Cell samples were incubated with the primary antibody for 1 h. The primary antibody solution was then removed and cells washed with PBS (5 times). Atto 488-labelled goat, anti-mouse IgG, diluted according to manufacturer's instructions in 1% HSA/PBS, was then applied to the cells for 1 h. This was then aspirated and cells

washed with PBS extensively. The Transwell[®] filter was excised and mounted on glass slides for confocal imaging as above.

Permeability characteristics of decellularised substrates

Decellularisation of permeable supports following Calu-3 culture was performed as described previously (section 'Caco-2 culture on decellularised substrates'). Material translocation across decellularised substrates was assessed by applying macromolecules and nanoparticles in HBSS on the donor chamber and sampling the basolateral solution periodically (every 30 min) for 3 h, whilst replacing the sampled volume. FD4 and FITC-labelled HSA were applied at 500 µg/ml, whilst FITC-labelled IgG was used at 80 µg/ml. Fluorescently-labelled, carboxy-functionalised polystyrene nanoparticles of 20 and 50 nm diameter were applied at 200 µg/ml. Hydrodynamic diameters of dextran, HSA and IgG were determined by DLS.

Results

Expression of laminin-5 by Calu-3 cells

Fig. 1 shows confocal micrographs of polarised Calu-3 cells cultured using serum-free conditions and immunostained for laminin-5 ($\alpha 3\beta 3\gamma 2$) as a BM marker. The presence of fluorescence signal (appearing in green), indicative of protein expression, is apparent in cell samples incubated with both primary and secondary antibodies (Fig. 1A and B). The distribution pattern of fluorescence across the depth of the polarised cell layer, as shown in the 'gallery' series of micrographs reflecting the apical-to-basolateral serial imaging on the cell monolayer (Fig. 1A), as well as in the 'three dimensional' image (Fig. 1B, reconstituted from multiple vertical plane sections) reveals a predominantly basolateral localisation of fluorescence. A control experiment whereby cells were treated with the secondary antibody only was conducted to ensure that the fluorescence is not an experimental artefact; in this instance fluorescence signal was largely absent (Fig. 1C).

Substrate protein deposition by Calu-3 cells

Following the observation that Calu-3 cells express laminin-5 as BM marker, further experiments probed whether this protein is deposited by the cells on the culture plastic surface. Initial experiments determined protein deposition on the filters arising after culture of Calu-3 cells as polarised layers for different time periods followed by decellularisation. Quantitation of substrate deposited proteins was possible after protein desorption via substrate incubation and washing with high ionic strength sodium chloride. The data in Fig. 2A shows that Calu-3 cells deposit approximately 150–300 ng protein/cm² following their culture as polarised layers. Surprisingly, a lower amount of deposited protein (150 ng/cm²) was detected following a longer culture period (19 days) relative to that associated with a shorter culture time (300 ng/cm² following a 5 day culture).

Immunostaining of decellularised filter plastic for laminin-5 shows a positive fluorescence signal for this protein (Fig. 2B i), unlike a control experiment where no notable fluorescence was observed following a laminin-5 immunostaining step of plastic

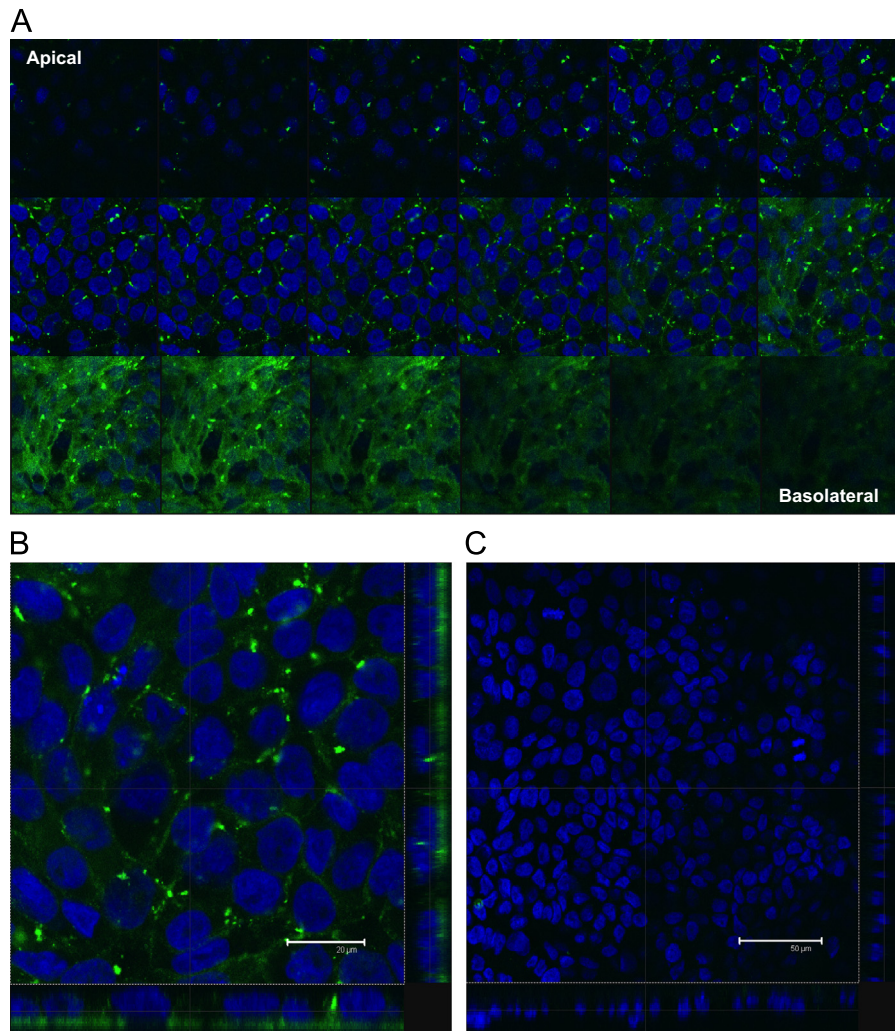


Fig. 1 – Expression of laminin-5 by Calu-3 cells. Cells were cultured as polarised layers in air interface culture and were treated with mouse anti-human laminin $\alpha 3$ /Laminin-5 primary antibody, followed by goat, anti-mouse IgG-Atto 488 secondary antibody (green). (A) ‘Gallery’ series of images (‘slices’), starting from the apical side of the cell layer and terminating at the basolateral side. (B) Orthogonal slices view showing the cell layer in ‘3D’, reconstructed from a series of vertical sections. (C) Immunostaining, where incubation with the primary antibody was omitted. Cell nuclei (blue) were labelled with Hoechst 33342.

filter supports exposed to the Calu-3 culture medium only, without cells (Fig. 2B ii).

Caco-2 culture on decellularised substrates

Cell attachment

To determine whether BM deposited on substrates influences epithelial cell behaviour, we employed Caco-2 cells and assessed their attachment and growth, in addition to barrier characteristics and morphology. This was possible as the serum-free conditions used in this work were found to severely inhibit these cells’ capacity to attach to surfaces. This is evident from Fig. 3, where poor cell attachment is apparent from images. A largely bare porous plastic support with limited areas of patchy cell growth (white circle) can be seen in Fig. 3A. This pattern is further confirmed when imaging the cells by SEM (Fig. 3B) and confocal microscopy (Fig. 3C). Furthermore, the expression of laminin-5, as

a BM component that regulates cell anchorage [22] and is clearly present in Calu-3-deposited BM, by Caco-2 cells is reported to be either very low or non-existent [23,24],

Serum-free culture of Caco-2 cells on Calu-3 decellularised supports (having BM proteins produced by Calu-3 cell layers) produced an entirely opposite outcome in terms of cell attachment. In this instance Caco-2 cells completely covered the substrate, such that the cell-covered porous support appears opaque rather than clear (Fig. 3D). Fully cell populated areas were also observed when imaging the cells by microscopy (SEM and confocal, Fig. 3E and F, respectively).

Electrical resistance properties

Since Caco-2 cells cultured on decellularised substrates demonstrated good cell attachment (Fig. 3), the current experiment determined the monolayers’ barrier in terms of TEER. An incomplete coverage of the growth area when cells were cultured on

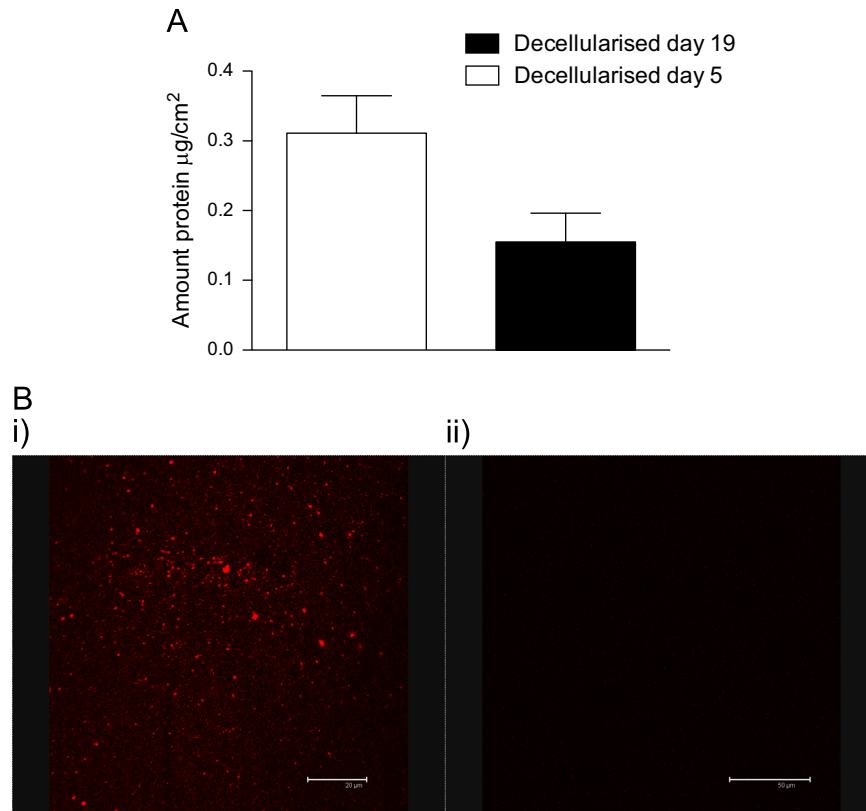


Fig. 2 – Deposition of basement membrane protein on growth substrates by Calu-3 cells. (A) Quantitation of Calu-3 surface deposited protein by bicinchoninic acid (BCA) assay following cell culture as polarised layers for 5 or 19 days and decellularisation. Adsorbed protein was desorbed by incubation with 8 M NaCl. (B) Immunofluorescence of Transwell® porous support following Calu-3 culture as polarised layers and decellularisation. (i) Decellularised supports were treated with mouse, anti-human laminin $\alpha 3$ /Laminin-5 primary antibody, followed by goat, anti-mouse IgG-Atto 488 secondary antibody (pseudocoloured red). (ii) Treatment of decellularised supports with the secondary antibody (incubation with the primary antibody was omitted).

unmodified filters is reflected in low TEER values, which do not increase beyond $30 \Omega\text{cm}^2$ even after allowing a 22-day culture period (Fig. 4A). Culture of Caco-2 cells under serum-free conditions on collagen coated filters improved the TEER profile, with values peaking at $700 \Omega\text{cm}^2$ on day 11, but high TEER was not sustainable and dropped rapidly after this point (Fig. 4B). Fibronectin-coated supports did not improve the TEER of serum-free Caco-2 cells. When cultured on Calu-3 decellularised substrates, Caco-2 cells displayed a typical TEER profile of increasing values with time in culture, followed by a plateau (Fig. 4C). Cells expressed a high maximal TEER value, reaching up to $2200 \Omega\text{cm}^2$ on day 15 of culture. TEER thereafter slightly decreased, though still remained at $>1700 \Omega\text{cm}^2$ until the last measurement time point (day 22).

Effect of BM protein inactivation on cell attachment

Caco-2 cells clearly display a remarkably improved attachment on decellularised substrates and this is most likely due to the surface-adsorbed, Calu-3-derived BM. To confirm that it is indeed a BM protein component that is responsible for this effect, decellularised substrates were treated with protein-damaging chemicals or BM protein digesting enzymes prior to Caco-2 cell seeding. The data in Fig. 5 shows that treatment of decellularised supports with trypsin did not markedly affect cell attachment, as indicated by a non-

remarkable ($<20\%$) reduction of TEER—employed as an indication of cell attachment (Fig. 5A). However, exposure of decellularised plastic filters to 4 M guanidine hydrochloride, a chemical that extracts BM components [25–27], significantly impaired the ability of Caco-2 cells to attach to filters, as noted by their TEER value of only 2.3% of control decellularised filters (treated with PBS). A similar outcome (TEER amounting to 2.8% of control) was apparent with treatment using 0.05 M NaOH, which was previously shown to inhibit the proliferation of endothelial cells when employed to treat BM-coated dishes [28]. Treatment of decellularised substrates with collagenase also notably affected the capacity of Caco-2 cells to form ‘electrically-tight’ monolayers, indicated by a low TEER of 5.3% relative to control and attach to decellularised substrates, as indicated by incomplete coverage of the growth area by the cell monolayer (Fig. 5B).

Morphological characteristics

Fig. 6 compares the morphology of conventionally cultured Caco-2 cells (unmodified supports and using serum-containing media) with cells of the same passage number, but cultured on decellularised supports. Scanning electron micrographs (Fig. 6A) show notably larger abundance of microvilli in the latter conditions (Fig. 6A i) compared to conventionally-cultured Caco-2 cells (Fig. 6A ii). Confocal micrographs also reveal differences in cell appearance between the tested conditions, with prominent

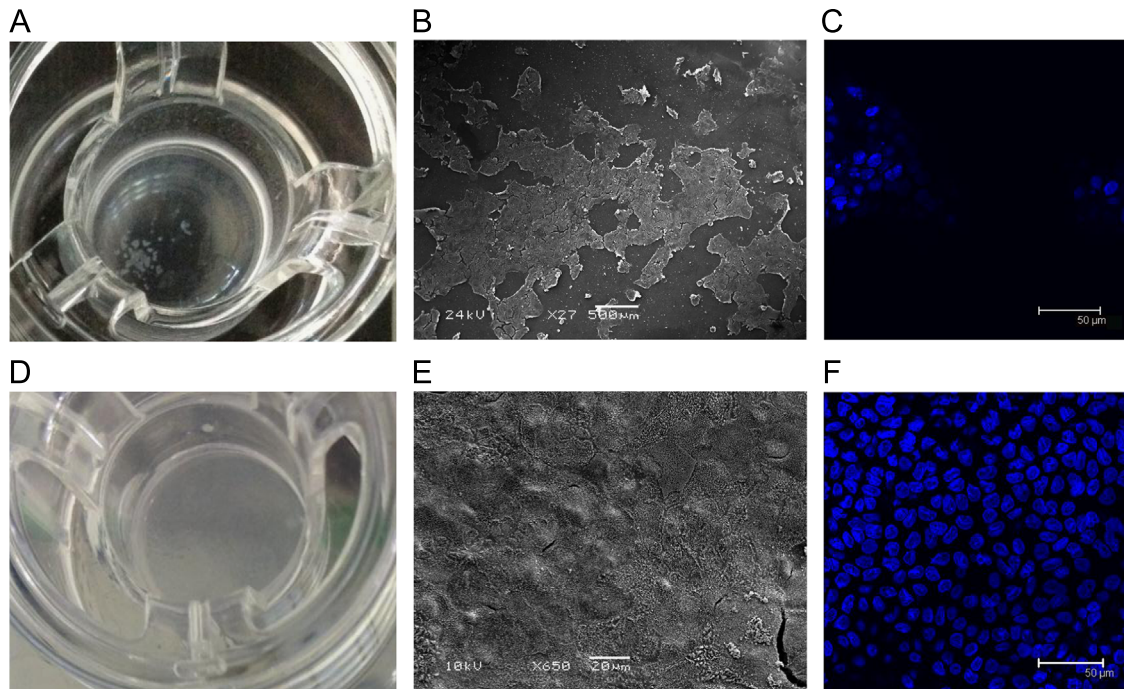


Fig. 3 – Effect of basement membrane on Caco-2 culture. (A)–(C) Serum-free culture of Caco-2 cells on unmodified porous supports. (D)–(F) Serum-free culture of Caco-2 cells on basement membrane-deposited supports, obtained via Calu-3 culture and decellularisation. (A) and (D) Photograph of Transwell® insert, (B) and (E) Scanning electron micrographs, (C) and (F) Confocal microscopy images. Caco-2 cells were cultured for 21 days in all cases. Culture conditions (e.g. medium and medium exchange rate) were kept the same for both conditions. Cell nuclei (blue) were labelled with Hoechst 33342.

differences in ZO-1 tight junction protein distribution (Fig. 6B). More specifically, the differences lie in the location of the tight junctions within the vertical plane of polarised cells, depicted on the bottom and right side of the micrographs, as well as their 'belt-like' distribution around the cells. Caco-2 cells cultured on BM-coated porous supports display a more prominently columnar morphology, with micrographs clearly depicting ZO-1 distribution considerably above the level of cell nuclei and appearance of the protein largely as smooth/linear connection between the cells (Fig. 6B i). On the other hand, cells cultured under conventional culture display a flatter morphology, with tight junctions appearing at the level of cell nuclei and having a largely convoluted arrangement (arrows) at cell–cell contacts (Fig. 6B ii).

Staining of Caco-2 monolayers for laminin-5 following their culture on decellularised supports is shown in Fig. 6C. Under these conditions, the cell monolayers stained positive for laminin-5, with prominent staining displayed on the basolateral side of the cells and at the level of the plastic porous support (Fig. 6C i). This is in contrast to staining of cells cultured on unmodified supports in the presence of serum (Fig. 6C ii), where the fluorescence signal is largely absent in the micrograph, suggesting lack of laminin-5 expression in Caco-2 monolayer growth using conventional culture.

Permeability characteristics of decellularised substrates

Decellularised substrate-cultured Caco-2 monolayers displayed a low and molecular weight-dependent permeability to two model

macromolecules (Fig. 7A). The permeability of two dextrans, FD4 (~4 kDa) and FD10 (~10 kDa), amounted to 9.7 and 7.6×10^{-8} cm/s. As the BM may contribute to this barrier, we carried out further experiments to examine whether Calu-3-originating BM coating the filter plastic hinders the movement of materials. A series of experiments assessed the diffusion of macromolecules and nanoparticles across decellularised porous supports. FD4, HSA and IgG were employed as macromolecules having a range of molecular mass, from ~4 kDa (FD4) to 66 kDa (FITC-HSA) to ~150 kDa (IgG). These results are presented as a graph of material movement (flux) against hydrodynamic molecular or nanoparticle size, determined by dynamic light scattering, in Fig. 7B. A significant pattern emerged from the data. Diffusion across decellularised supports was lower compared to unmodified supports for all of the tested materials and the extent of this difference increased with the size of the macromolecule or nanoparticle. For example, FD4 flux amounted to 0.31 and 0.23 $\mu\text{g}/\text{min}/\text{cm}^2$ across unmodified and decellularised supports, respectively, and the translocation of HSA across unmodified supports was 1.6-fold higher compared to decellularised supports (0.138 and 0.085 $\mu\text{g}/\text{min}/\text{cm}^2$, respectively). Decellularised porous supports were especially restrictive to the transport of FITC-IgG, with fluxes of 0.029 versus 0.007 $\mu\text{g}/\text{min}/\text{cm}^2$ in unmodified and decellularised Transwell® supports, corresponding to a 4.1-fold difference. Polystyrene nanoparticles of 20 nm traversed the unmodified supports at a rate that was 9.5-fold higher relative to decellularised supports (0.052 and 0.006 $\mu\text{g}/\text{min}/\text{cm}^2$, respectively). For 50 nm nanoparticles a larger effect was apparent, with

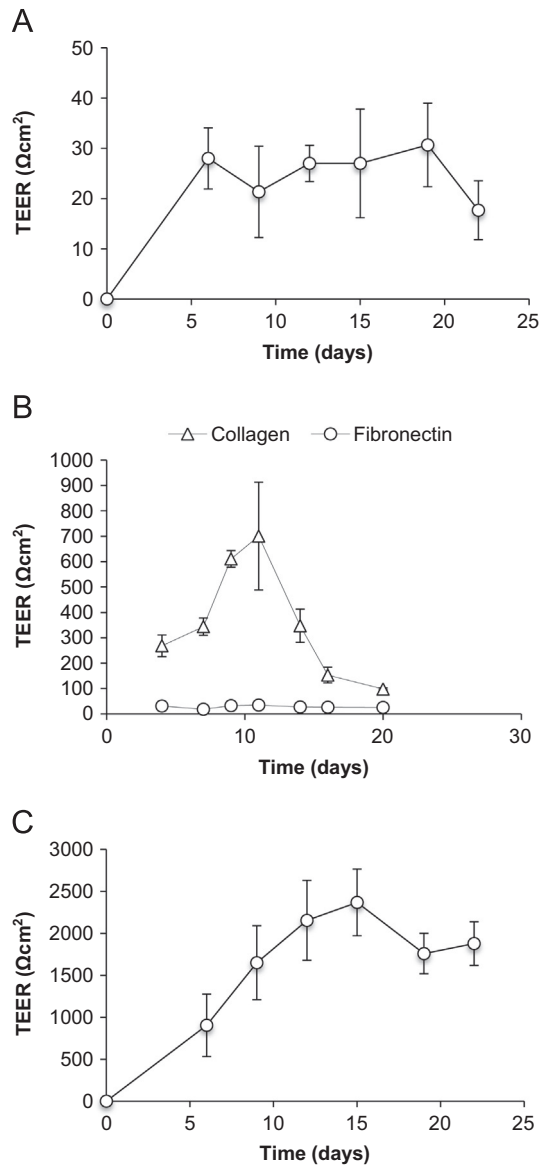


Fig. 4 – Transepithelial electrical resistance (TEER) of Caco-2 cells cultured on differently modified Transwell® supports using a serum-free medium. (A) Unmodified supports, exposed to Calu-3 medium (no cells) and 0.02 M ammonium hydroxide before cell seeding. (B) Human collagen IV and human fibronectin-coated supports. (C) Decellularised supports coated with basement membrane originating from Calu-3 cells. Caco-2 cells were seeded and maintained in culture using the same conditions in all instances (10^5 cells/cm² seeding density and using the same medium, which was replenished at the same time intervals in all cases).

unmodified supports showing a 31-fold higher nanoparticle translocation compared to decellularised counterparts.

Discussion

The BM is a dynamic structure that not only physically supports the epithelium but is essential in orchestrating and controlling a

variety of biological phenomena, including migration, proliferation, and differentiation of intestinal cells [13]. Epithelial cell-BM interactions in the intestinal epithelium are complex and this complexity is necessary considering its physiological function. The phenotype and function of small intestinal epithelial cells varies with position relative to crypt-villus structure and the cell-BM interaction plays an important role in influencing this function. For example, at the villus tip, intestinal epithelial cells are repeatedly exposed to a variety of foreign bodies, undergoing exhaustion and/or injury. Within this region, the shedding of epithelial cells into the lumen [29,30] is therefore important for maintaining continual epithelial renewal and hence epithelium function and integrity [31]. This renewal is achieved by migration of the epithelium along the BM from the proliferative undifferentiated segments in the crypts to the tips of the villi. Epithelial cell migration is in turn thought to be possible due to changes in BM-integrin interactions, which may also reinforce E-cadherin-dependent cell–cell adhesion [32], along the crypt-to-villus axis, reflecting the changing BM composition along this axis (which parallels the differentiation process of epithelial cells).

The Caco-2 cell line is currently the ‘gold standard’ in vitro intestinal epithelial model. These cells are typically cultured on flat, porous supports and after a certain culture period can spontaneously differentiate into polarised small intestinal epithelial cell-like phenotype. Whilst the Caco-2 model plays an important role in in vitro research related to the intestinal epithelium, including drug absorption studies, this model lacks the expression of some proteins such as drug metabolizing enzymes [33,34] and fails to express late markers of differentiation, such as apolipoproteins and proteins involved in lipid metabolism [35–37]. Employing flat, porous supports as substrates for Caco-2 culture is a convenient approach avoiding the need to reproduce the complex biological environment with the folded crypt-villi architecture and the BM micro-milieu. However, such flat substrates that lack the BM do not represent the physicochemical situation of the human intestine and may compromise the physiological relevance and performance of the in vitro model. Furthermore, the lack of more sophisticated substrates that represent the growth environment of the intestinal epithelium explains the so far unsuccessful attempts to routinely maintain primary enterocytes in culture for prolonged intervals [38].

This study employed a simple approach to obtain a biologically derived, epithelially-sourced BM deposited on cell culture substrates. In this respect an airway epithelial cell line was used to produce the BM, with these cells cultured and allowed to assemble a BM, followed by their lysis. This set up was adopted upon the observation that these (Calu-3) cells prominently express laminin-5 (Fig. 1A), used here as a BM marker, with evidence that this protein was in fact deposited on filter substrates (Fig. 1A and Fig. 2). Indeed, the approach of obtaining BM coated substrates that are naturally assembled by cells in vitro has been reported before. BM deposited by Madin–Darby canine kidney (MDCK) epithelial cells, which was obtained in a similar manner as our work (by decellularisation), was used in work determining the glomerular permeability to macromolecules [39].

We selected laminin-5 as a marker for the BM in the view of its wide distribution in the BMs of epithelial tissues, including its presence in the villus cells of the normal human small intestinal epithelium [40,41] and its role in migration and differentiation of

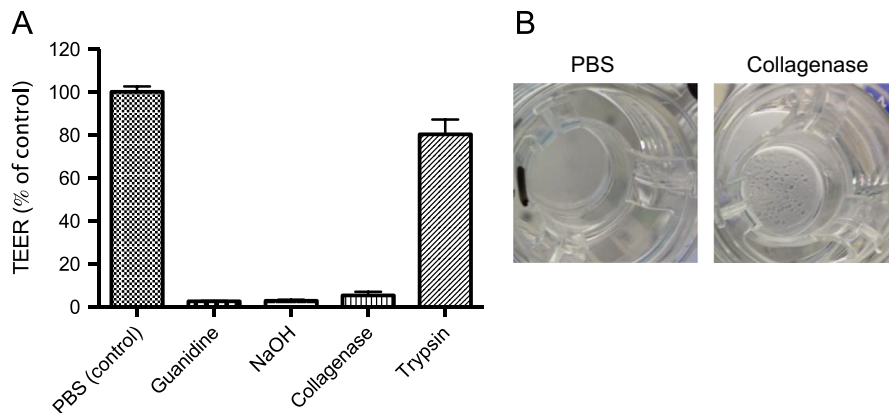


Fig. 5 – Effect of chemical and enzymatic treatment of decellularised supports on Caco-2 transepithelial electrical resistance (TEER) (A) and cell attachment (B). Transwell® porous supports were initially used for culture of Calu-3 cells, which were then decellularised. Following decellularisation and washing with PBS, supports were treated with PBS (control), 4 M guanidine hydrochloride, 0.05 M NaOH, 1 mg/ml collagenase or trypsin for 1 h. Supports were then washed multiple times with PBS before Caco-2 seeding. Caco-2 cells were maintained thereafter in the same serum-free medium in all cases. TEER measurements and photographs were taken after a 21-day culture on permeable supports.

intestinal epithelial cells [41]. Laminins (more specifically, their α chains) are recognised by cell surface receptors, such as integrins [22], and it is thought that the different extents of adhesion displayed by various laminin isoforms reflect the integrin repertoire of the cells [42]. Laminin-5 specifically is present in the human intestine, displaying a pattern of an increasing gradient from the upper crypt to the villus tip in the human small intestine [41,43], which suggests a possible role in differentiation and/or migration of intestinal cells [24]. This laminin subtype is thought to regulate the anchorage and motility of epithelial cells through various integrins [22]. However, the expression of laminin-5 by Caco-2 cells is reported to be either very low or non-existent [23,24], which is also confirmed following conventional culture (serum-containing medium and growth on unmodified porous supports) in our work (Fig. 6c ii).

Conversely, our data suggests that the airway epithelial Calu-3 cells clearly express laminin-5 and its presence in BMs of stratified epithelia, such as those of the airways, has also been shown previously [23]. Assembly and growth substrate deposition of BM by bronchial epithelial cells was also demonstrated previously with BEAS-2B cells [44].

In experiments examining substrate deposition of BM by Calu-3 cells, we cultured these cells for different time periods, followed by decellularisation, desorption of surface-adsorbed BM proteins and, finally, quantitation. Presence of protein on the supports was evident, with the amount of the deposited protein amounting between 150 and 300 ng/cm², depending on the point when decellularisation was performed (Fig. 2). This is similar to the protein density of 150 ng/cm², used previously for coating of substrates with reconstituted EHS matrix and shown to provide maximal hepatocyte attachment [45,46]. Interestingly, it was noted that a larger amount of deposited protein was apparent with an earlier Calu-3 decellularisation (day 5 of culture), which is a practical advantage.

Serum-free cultured Caco-2 cells used in the work demonstrated a greatly diminished ability to attach to plastic substrates compared to the same cells (same batch/passage of cells) when cultured under typically used, culture conditions with serum. The

data clearly demonstrated impaired Caco-2 cell attachment when plated on unmodified supports in serum-free medium, a phenomenon that is apparent to the naked eye (Fig. 3A), on examination by microscopy (Fig. 3B and C) and when measuring TEER (Fig. 4A). Monolayer culture of Caco-2 cells under these conditions was not successful (cells did not display a high and sustainable TEER) even after coating the substrates with collagen or fibronectin (Fig. 4B). On the other hand, presence of BM as a cell substrate produced a remarkable effect on Caco-2 attachment. Indeed, Fig. 3D and E show a complete substrate surface coverage by cells, indicating that the markedly impaired cell attachment (due to the lack of serum) is reversed on decellularised substrates. In addition to the micrographs, further experiments confirmed the cell attachment and cell monolayer integrity by way of TEER measurement and permeability to model macromolecules. Caco-2 cells cultured on decellularised, BM-coated filters displayed a TEER profile that is typical for conventional Caco-2 culture, with TEER increasing with time in culture until it reaches high values (up to > 2200 Ω /cm² in our experiments) (Fig. 4C). These monolayers also displayed a molecular weight dependent barrier to the permeability of two dextrans (Fig. 7A). Interestingly, the TEER and barrier characteristics of cells cultured under these conditions were similar to those of Caco-2 cells cultured with serum on unmodified supports, previously reported by our group [15].

The attachment and growth behaviour of serum-free cultured Caco-2 cells when plated on decellularised substrates is dramatically different to culture on unmodified supports and we attribute these effects to the surface adsorbed-BM (or BM components) previously assembled by airway epithelial cells, as shown with laminin-5 immunostaining. Indeed, this was probed in a series of experiments where decellularised porous supports were treated with different chemicals, having various effects on BM proteins, prior to Caco-2 cell seeding. Whilst trypsin did not affect cell attachment, Caco-2 cell attachment was remarkably suppressed (cells failed to develop a high TEER) by a range of other treatments. These were BM protein-extracting guanidine, a chaotropic agent which denatures and extracts proteins [25–27], sodium hydroxide used at a concentration to hydrolyse glycosidic

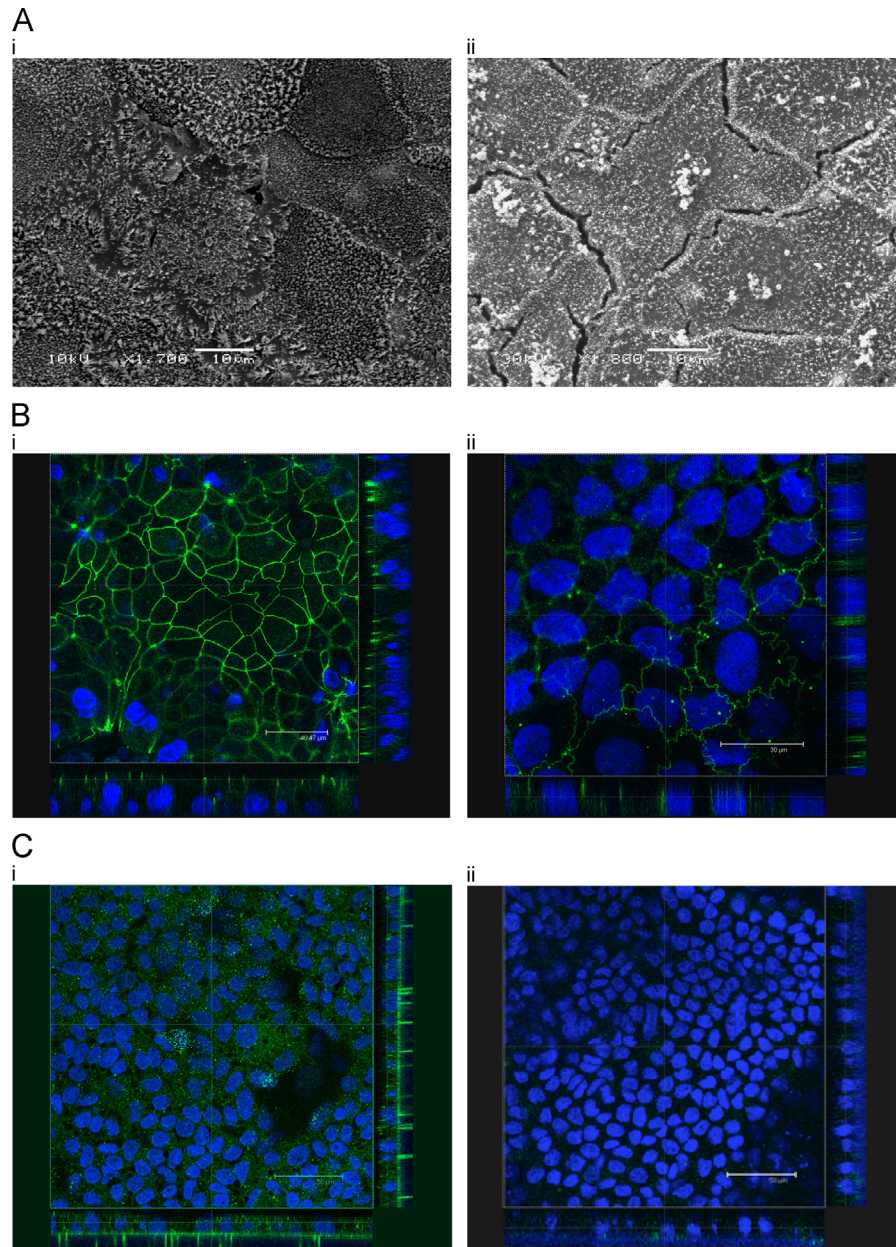


Fig. 6 – Effect of basement membrane on Caco-2 cell morphology. (A) Scanning electron micrographs of (i) cells cultured on decellularised Transwell® porous supports and (ii) cells cultured on unmodified supports. (B) Zonula Occludens-1 (ZO-1) tight junction protein immunostaining of (i) cells cultured on decellularised supports and (ii) cells cultured on unmodified supports. ZO-1 immunostaining conducted by cell treatment with mouse, anti-human ZO-1 (primary) antibody, followed by goat, anti-mouse Atto 488-IgG. C) Laminin-5 immunostaining of (i) cells cultured on decellularised porous supports (serum-free), and (ii) cells cultured on unmodified supports (serum-containing medium). Immunostaining conducted using mouse, anti-human laminin α 3/Laminin-5 primary antibody, followed by goat, anti-mouse IgG-Atto 488 secondary antibody (green). Cell nuclei (blue) were labelled with Hoechst 33342.

linkages to sugars and the extracellular matrix-degrading enzyme collagenase. Although full characterization of the Calu-3-deposited BM was not conducted in this work, our data suggest a rich laminin-5 presence. This is a key BM protein that displays intestinal localization that is variable according to the crypt-to-villus positioning [41,43,47,48] and has a role in differentiation and/or migration of intestinal cells [24]. An earlier publication comparing cell attachment to different laminin isoforms revealed

that Caco-2/TC7 cells bound more avidly to laminin-5 and laminin-10 compared to laminin-1 or laminin-2 [42].

The effect of BM substrates on Caco-2 cells was not limited to cell attachment. Examining the morphology of cells cultured on decellularised supports and drawing a comparison with Caco-2 cells cultured on untreated surfaces in the presence of serum, a striking difference between the two conditions was apparent in terms of cellular architecture and the abundance of microvilli

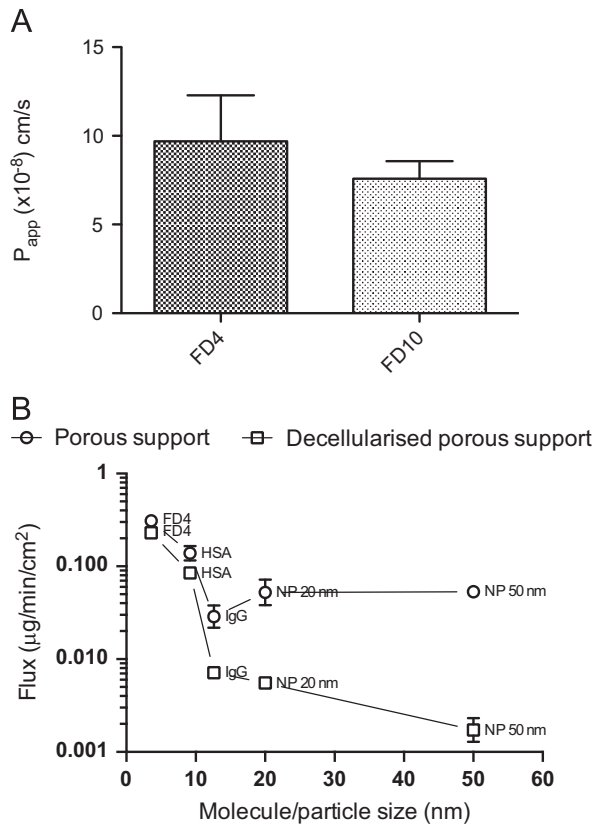


Fig. 7 – Permeability of macromolecules and nanoparticles across different barriers. (A) Permeability of FITC-dextran of 4 and 10 kDa (FD4 and FD10, respectively) across Caco-2 monolayers cultured on decellularised substrates. Substrate decellularisation was conducted by treating Calu-3 cells with 0.02 M ammonium hydroxide and washing with PBS. FD4 and FD10 were applied in Hank's Balanced Salt Solution at 500 μ g/ml. Permeability is expressed as apparent permeability coefficient (P_{app}). (B) Diffusion of macromolecules and nanoparticles across Calu-3-deposited basement membranes. Transwell® porous supports were used to culture Calu-3 cells as polarised layers, followed by decellularisation. The supports were washed thoroughly with PBS before the permeability study. Hank's Balanced Salt Solution was used as the transport solution. FD4 and Human Serum Albumin (HAS) were applied at 500 μ g/ml, whilst human serum FITC-labelled IgG was used at 80 μ g/ml. 20 and 50 nm nanoparticles were applied at 200 μ g/ml in HBSS. Material diffusion is expressed as flux. Material transport was measured by applying the samples on the apical chamber and sampling the basolateral solution every 30 min for 3 h and quantitation of samples by fluorescence (through calibration curves) in all cases.

(Fig. 6A). The significantly more abundant microvilli on the apical membranes of Caco-2 cells cultured on decellularised filters suggest a well-developed brush border and a higher level of differentiation [49–51].

Another morphological aspect that was clearly influenced by growth on BM-deposited substrates was the tight junction. Both the localisation along the apical-to-basal axis of polarity and the 'belt-like' appearance of the tight junction protein, ZO-1, were notably different in Caco-2 cells grown on decellularised filters

(Fig. 6B). The increased height of the tight junctions gave rise to a prominently columnar appearance of cells under these conditions, as compared to a flatter monolayer appearance of Caco-2 cells cultured on untreated substrates (with serum). Columnar appearance of Caco-2 cells suggests a higher level of differentiation. This morphological criterion was in fact used previously as a marker of differentiation whereby the x–y (lateral) surface area measurements were used (indicating how columnar the cells are) as a measure of differentiation [51]. An epithelial monolayer having a more prominent columnarity also more closely resembles the native human small intestinal epithelium [52]. Equally, the smooth almost linear distribution of the tight junctions achieved with Caco-2 cells cultured on BM-deposited substrates is also a closer representation of natural intestinal epithelium than the convoluted appearance noted with conventional culture reported in our earlier work [15] and also in publications by others [53–55].

The effect of BM on Caco-2 morphology is perhaps understandable considering that epithelial cell–BM (as well as cell–cell) adhesion systems are linked to the cytoskeleton, which controls cell polarization and the interaction between BM/ECM and integrin induced cytoskeletal rearrangements [56,57]. Cell adhesion to the BM therefore contributes to the apical-to-basal axis of polarity, in vivo as well as in vitro [32]. Staining of Caco-2 monolayers for laminin-5 following culture on decellularised filters signalled its presence on the basolateral plane of the cells (Fig. 6C ii), unlike the staining of cells cultured on unmodified supports (Fig. 6C i).

The positive effect of BM on Caco-2 differentiation was also demonstrated previously. A study by Le Beyec et al. showed Caco-2 differentiation was boosted by the presence of basal lamina of mesodermal origin [58]. The influence of exogenous laminin substrates on the differentiation state of Caco-2 cells has also been shown [13,47], with Basson et al. reporting that laminin stimulates Caco-2 expression of four different brush border enzymes (alkaline phosphatase, dipeptidyl peptidase, isomaltase and lactase) and these effects were greater for laminin (for all four brush border enzymes) than for type IV collagen. However, other work in the area tends to employ individual ECM components for substrate coating [59,60] rather than the biologically-important heterogeneous BM.

The data overall show that BM-deposited substrates, assembled by an airway epithelial cell line, showed a remarkable effect on Caco-2 attachment and morphology, with the latter suggesting a higher level of differentiation. Whether the culture of Caco-2 cells on decellularised supports induced the synthesis and assembly of some of their own BM or Caco-2 cells merely used the Calu-3-assembled/deposited BM to interact with is not clear. In any case, the work shows that the presence of BM on substrates not only completely reverses the ability of Caco-2 cells to adhere to the surface, which is lost upon serum free culture, but also promotes a higher level of differentiation. Both these points are important in terms of in vitro Caco-2 culture. The use of serum-free media is advantageous as it potentially removes a major source of Caco-2 model variability (serum of undefined composition can vary markedly from batch to batch) and it provides a more appropriate environment for drug transport studies due to the absence of apical serum in the intestinal epithelium. However, the culture of Caco-2 cells as polarised/differentiated monolayers in the absence of serum is challenging and, as a result, not widely reported.

A few studies that have described this have used highly complex and expensive media recipes [61,62] or commercially available preparations such as MITO+ serum extender [63]. The culture conditions in our study allowed us to use a relatively simple and inexpensive serum-free (and animal component free) medium. The differentiation aspect is of significance as achieving a higher level of cell differentiation *in vitro* would enhance the relevance of the model due to a closer resemblance to the native tissue.

Importantly, our work considered the potential drug delivery barrier of the BM. From the drug delivery perspective this is a valuable investigation as it is an unexplored area. This part of the work assessed the translocation of macromolecules of a range of molecular weights, as well as model nanoparticles of two sizes, across decellularised supports (with airway epithelium-deposited BM). The data demonstrated that the movement of both macromolecules and nanoparticles across the decellularised porous supports was hindered, with dramatic effects observed for larger macromolecules, as exemplified by the lower permeability of IgG, the lower level of translocation for 20 nm nanoparticles and a dramatic reduction in transport for 50 nm-sized particles. This is particularly important result in relation to biotherapeutics and nanomedicines, which are becoming increasingly important and where delivery via the oral route (i.e. non-invasively) is of high interest. It is clear therefore that the BM needs to be taken into consideration in the transport of a variety of materials across epithelia. The presence of a similar barrier at endothelia suggests that similar considerations will apply to transport of macromolecules and particles across this barrier.

Our results on the barrier effect of BM support a range of observations reported in the literature. The BM of nonkeratinized oral mucosal epithelium has been shown to limit the movement of a bacterial endotoxin [64] and 2',3'-dideoxycytidine [65], a relatively small mass (211 g/mol) ionic compound. BM hydrogels were also shown to suppress the diffusive motion of HPV-16 pseudovirions by as much as ~10,000 times compared to pseudovirion diffusion in buffer control [66]. The latter study also reported that the lining of a HeLa monolayer with BM followed by exposure to HPV-16 pseudoviruses reduced the percentage of infected HeLa cells by about 6-fold, highlighting the extent of the barrier that the BM presents to the movement of a 50 nm virus. The ECM has been shown to drastically suppress the diffusion of both positively and negatively charged particles, even when the particles are significantly smaller than the mesh size of the ECM [67]. The same may therefore apply to the BM. A major contributor to this barrier property of the ECM was shown to be the heparan sulfate, a strongly anionic linear polymer of uronic acid and glucosamine disaccharide units as the barrier function of the ECM is lost following enzymatic digestion of this component.

Our findings may have wider implications in the field of epithelial cell culture in general, potentially enabling growth of 'difficult to culture' cells, such as weakly adherent cells and primary cells, in addition to improving the existing *in vitro* intestinal epithelial models. This improved serum-free culture of Caco-2 cells, which produces a model of closer morphological resemblance to the native epithelium is also convenient. The BM in this work is naturally assembled by an epithelial cell line following a short (5-day) culture and is an inexpensive approach to obtain more biorelevant substrates compared to commercially available alternatives such as the BD Biocoat™ inserts (coated with a single BM component, fibrillar collagen) and BM extracts,

which are animal-derived and contain non-BM components such as growth factors and enzymes [68]. Caco-2 culture on a BM 'blanket' left behind by a different epithelial cell source is also advantageous as it provides a physiologically relevant, heterogeneous BM as compared to the use of individual BM components, which may not be able to support serum-free Caco-2 culture, as shown for type IV collagen and fibronectin (Fig. 4B), or mixtures of BM components in non-physiological proportions. When compared to commercially available BM extracts, we anticipate that the microporous substrates in our work contain a BM of physiological thickness (based on the amount of adsorbed protein, Fig. 2A), which is important for epithelial cell growth. BM extracts such as Matrigel are typically used as physiologically irrelevant thick layers [69], which is inappropriate for drug transport studies.

Conclusion

This study overall shows that the cell attachment capacity of Caco-2 cells, which is completely lost upon serum-free culture, is fully restored when the cells are grown on a BM and that cells cultured in this manner more closely resemble the native intestinal epithelium. Whilst the BM plays a significant role on cell attachment and morphology, we present evidence that this protein network also notably affects the movement of macromolecules and nanoparticles.

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